An atypical member of the light-harvesting complex stress-related protein family modulates diatom responses to light

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Diatoms are prominent phytoplanktonic organisms that contribute around 40% of carbon assimilation in the oceans. They grow and perform optimally in variable environments, being able to cope with unpredictable changes in the amount and quality of light. The molecular mechanisms regulating diatom light responses are, however, still obscure. Using knockdown Phaeodactylum tricornutum transgenic lines, we reveal the key function of a member of the light-harvesting complex stress-related (LHCSR) protein family, denoted LHCX1, in modulation of excess light energy dissipation. In contrast to green algae, this gene is already maximally expressed in nonstressful light conditions and encodes a protein required for efficient light responses and growth. LHCX1 also influences natural variability in photoresponse, as evidenced in ecotypes isolated from different latitudes that display different LHCX1 protein levels. We conclude, therefore, that this gene plays a pivotal role in managing light responses in diatoms.


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Diatoms are prominent phytoplanktonic organisms that contribute around 40% of oceanic organic carbon production and to constitute an important component of the biological carbon pump (1, 2). They are widespread throughout the oceans and show optimal photosynthetic activity over a wide range of environments (3–5). Their extreme flexibility to changing conditions is thought to be a key element that has driven their rise to dominance in contemporary oceans (6). For example, they show an outstanding capacity to cope with light stress. Their ability to dissipate excess energy in high light can surpass that of plants, as witnessed by the impressive accumulation of nonphotochemical quenching (NPQ) in green algae, refs. 13, 14), and hereafter named LHCSR in this study (Fig. 1). Genes encoding these proteins are absent in higher plants but are induced by photooxidative stress in some unicellular photosynthetic organisms (Fig. S1 and refs. 13, 14). Their accumulation upon light stress has been associated with enhanced photoprotection in the green freshwater alga Chlamydomonas reinhardtii (15). A similar role was recently proposed for diatom orthologs that are upregulated in high light (16–18). On the other hand, gene expression analyses revealed that one of the four LHCSR genes of P. tricornutum (LHCX1) is not light-stress responsive (Fig. 1). In cells grown under 12:12 h light:dark cycles, exposure to low irradiance (30–70 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}) already induced maximal LHCX1 induction, whereas expression of the other isoforms remained almost undetectable. LHCX1 mRNA levels remain stable even with increasing light intensities (Fig. 1C and ref. 17). Recent data have suggested that this isoform could not be responsible for NPQ responses, but would play instead a structural role within the PSII–fluoroxanthin chlorophyll protein (FCP) supercomplex (18). However, induction of this gene upon a dark-to-low light transition paralleled with an enhancement of the NPQ capacity (Fig. 1B), i.e., the maximum extent of energy dissipation in the pigment-containing proteins of photosystem (PS) II, whenever light absorption exceeds the maximum rate of CO\textsubscript{2} assimilation. In plants, NPQ relies on acidification of the luminal pH, which affects zeaxanthin-violaxanthin pigment composition via the xanthophyll cycle (XC) and the activity of the PSII subunit PsbS (8, 9). State transitions of the light-harvesting complexes between PSII and PSI add an additional layer of photoprotection (8). In diatoms, state transitions have not been found (10) and the PsbS gene is absent from the nuclear genomes sequenced to date (2). Instead, the superior capacity of diatoms for NPQ has been attributed to the existence of an alternative XC, also observed in other chromophytes (11), which catalyzes the de-epoxidation of diadinoxanthin (DD) to diatoxanthin (DT), the better studied cycle present in plants being observed only in high light grown diatom cells (12).

To test the existence of additional NPQ effectors in diatoms, expression of light stress response proteins was examined in P. tricornutum. In contrast with green algae, we found that LHCX1, one of the putative high light response genes of the LHCSR family of diatoms, is already maximally expressed in low light and that its gene product modulates NPQ capacity during a typical light/dark cycle. Transgenic lines with reduced expression of the gene have significantly reduced NPQ and growth capacities. Analysis of natural NPQ variants of P. tricornutum indicates a key role of LHCX1 not only in the transient response to light, but also in constitutive adaptation to environmental constraints.

Results

LHCX1 Is Required for Light Responses in Both Stress and Nonstressful Conditions. Genomic and phylogenetic analyses reveal in diatoms the presence of multiple members of the light-harvesting complex stress-related proteins LHCSR (also known as LHCSR/LH18 in green algae, refs. 13, 14), and hereafter named LHCX in this study (Fig. S1). Genes encoding these proteins are absent in higher plants but are induced by photooxidative stress in some unicellular photosynthetic organisms (Fig. S1 and refs. 13, 14). Their accumulation upon light stress has been associated with enhanced photoprotection in the green freshwater alga Chlamydomonas reinhardtii (15). A similar role was recently proposed for diatom orthologs that are upregulated in high light (16–18). On the other hand, gene expression analyses revealed that one of the four LHCSR genes of P. tricornutum (LHCX1) is not light-stress responsive (Fig. 1). In cells grown under 12:12 h light:dark cycles, exposure to low irradiance (30–70 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}) already induced maximal LHCX1 induction, whereas expression of the other isoforms remained almost undetectable. LHCX1 mRNA levels remain stable even with increasing light intensities (Fig. 1C and ref. 17). Recent data have suggested that this isoform could not be responsible for NPQ responses, but would play instead a structural role within the PSII–fluoroxanthin chlorophyll protein (FCP) supercomplex (18). However, induction of this gene upon a dark-to-low light transition paralleled with an enhancement of the NPQ capacity (Fig. 1B), i.e., the maximum extent of energy dissipation in the pigment-containing proteins of photosystem (PS) II, whenever light absorption exceeds the maximum rate of CO\textsubscript{2} assimilation. In plants, NPQ relies on acidification of the luminal pH, which affects zeaxanthin-violaxanthin pigment composition via the xanthophyll cycle (XC) and the activity of the PSII subunit PsbS (8, 9). State transitions of the light-harvesting complexes between PSII and PSI add an additional layer of photoprotection (8). In diatoms, state transitions have not been found (10) and the PsbS gene is absent from the nuclear genomes sequenced to date (2). Instead, the superior capacity of diatoms for NPQ has been attributed to the existence of an alternative XC, also observed in other chromophytes (11), which catalyzes the de-epoxidation of diadinoxanthin (DD) to diatoxanthin (DT), the better studied cycle present in plants being observed only in high light grown diatom cells (12).
accumulation in this strain. Reduced NPQ capacity always
Fig. S2 mRNA expression levels, and LHCX protein
are the maximum isoforms, mainly LHCX1 and LHCX2
17). This paralleled a significant increase in the accumulation
of LHCX proteins in the cell (Fig. 1D). Because LHCX1 always
remained the most abundant mRNA, this finding suggests possible
differences in translation efficiency and/or protein stability
between the different isoforms. High light exposure induced other
typical photoprotective responses (increased activity of the XC
and enhanced xanthophyll accumulation in the cells) (7, 12), ul-
timately leading to an additional (albeit small) increase in NPQ
(Fig. 1B), in agreement with previous studies (19). Altogether,
these data suggest that induction of LHCX proteins in high light
increases energy quenching as part of a more general photo-
protective response. Conversely, expression of the LHCX1 gene
in low light seems to provide P. tricornutum cells with nearly
maximum NPQ capacity, pinpointing the LHCX1 isoform as
a likely NPQ effector in diatoms.

Analysis of NPQ changes in diatoms exposed to a 12:12 h light:
dark cycle at low light (70 μmol photons m−2 s−1) further con-
ﬁrmed this observation. We observed that in low light, expression
of the LHCX1 gene slowly declined after its induction following
a dark-to-light shift. We found a tight correlation between NPQ
capacity, LHCX1 mRNA expression levels, and LHCX protein
abundance, contrasting with the stable XC activity (Fig. 2). This
suggests that LHCX1 actively participates in modulating the
extent of energy dissipation in P. tricornutum, for a given de-
epoxidation state (DES). This possibility was further investigated
by generating knockdown transgenic lines by RNA interference
RNAi). Three clones were identiﬁed, which showed reduced
expression of the LHCX1 gene and a consequently lower protein
accumulation (Fig. 3A). The transgenic lines showed a ~50% reduction in NPQ capacity (Fig. 3B), although their XC capacity
(Fig. 3A), PSII efﬁciency, maximal photosynthetic electron
flow, PSII absorption cross-section (Table 1), and pigment composi-
tion (Table S1) were the same as in wild-type cells, when mea-
sured in unstressed cells. This effect was specific, as no reduction
was conﬁrmed for the lhcx1a line (Fig. S2B), for which a reduced
NPQ capacity throughout the day correlated with the reduced
expression of LHCX1 and an unchanged DES (Fig. S2C). Whereas photosynthesis showed a similar light saturation profile
in the Pt1 and lhcx1a strains (Fig. S3A), the NPQ response was reduced throughout the whole light intensity range in the
silenced line. In particular, the finding that NPQ was smaller in the
lhcx1a even in conditions where LHCX isoforms other than
LHCX1 were induced (Fig. 1 and Fig S2) strongly suggests that
the expression of these genes cannot compensate for the lower
LHCX1 accumulation in this strain. Reduced NPQ capacity
paralleled with enhanced photoinhibition in lhcx1a cells, as evi-
denced by the declined oxygen evolution capacity measured
upon prolonged exposure to strong light (Fig. S3B).

The notion of a central role of LHCX1 in light acclimation in
diatoms was further reinforced by measuring the growth capacity
in wild-type and RNAi lines. Reduced growth was seen in the
three strains under nonstressful light conditions (Fig. 3C and Fig.
S4A), in high light (Fig. S4B), and also upon exposure to in-
termittent light (Fig. S4C), a condition particularly favorable for
the development of NPO in P. tricornutum (7). Because reduced
growth could not be accounted for by a modification of the overall
photosynthetic performance in unstressed cells (Table 1), we exclude possible pleiotropic effects of LHCX1 misregulation

![Fig. 1. Diatom LHCX expression and NPQ characteristics in dark, low light
and high light conditions. (A) De-epoxidation state (DES) determined as DT/
(DD + DT), and relative level of the pool of xanthophyll (DT + DD), normalized
to the fucoxanthin (Fx) content, obtained from HPLC analysis. (B) NPQ response
from cells grown as described above. NPQ was calculated as (Fm-Fm′)/Fm,
where Fm and Fm′ are the maximum fluorescence emission measured in dark
(30-min adaptation before exposure) and cells exposed for 5 min to 700 μmol
photons m−2 s−1, respectively. (C) Accumulation of the four P. tricornutum
LHCX transcripts determined by qRT-PCR from cells grown in a 12.12 h light:
dark cycle and collected in the dark (dark), after 2 h of low light treatment
(70 μmol photons m−2 s−1), LL, and after 1 h of a subsequent low light to high
light shift (600 μmol photons m−2 s−1), HL. RPS was used as reference gene.
Error bars are relative to three independent experiments. (D) Western blotting
showing LHCX protein accumulation. Proteins were detected with antibodies
against the LHCX/LHCSR and the PSII subunit D2 was used as loading control.

![Fig. 2. Diurnal changes in DES, NPQ, and LHCX transcript (A), and LHCX
protein levels (B). Cells were exposed to a 12:12 h light:dark regimen at 70
μmol photons m−2 s−1, and samples were collected at the indicated times
during the light phase for the different measurements. Error bars refer to
duplicate measurements in three biological samples. NPO, DES, and blot
analyses were performed as speciﬁed in Fig. 1.]
on photosynthesis. Conversely, the reduced fitness of lhcx1 strains suggests that LHCX1 could be important for optimum light utilization in diatoms in a wide range of photon fluxes.

**Natural NPQ Variants of P. tricornutum with Altered LHCX1 Expression.** Although *P. tricornutum* is generally considered to have only limited ecological relevance, it has been found at several locations worldwide (Fig. 4A and ref. 20). We used the available strains to screen for natural NPQ variants. Only minor differences were observed in most of them, with the significant exception of Pt4, the strain isolated from the highest latitude and therefore adapted to the lowest ambient light intensities (Fig. 4A and ref. 20). This ecotype displayed systematically reduced NPQ levels with respect to other strains in all conditions tested, including the low temperatures (Fig. 4A and Fig. S5) that characterize its natural environment (21). This suggests that Pt4 is a natural NPQ variant, most probably reflecting a constitutive adaptation to its environment.

In agreement with the results obtained from the lhcx1 RNAi lines, the reduced NPQ capacity in Pt4 did not correlate with a diminished XC capacity (Fig. 4B) or a reduced photosynthetic performance (Table 1), but could be linked to a specific decrease in the expression of the LHCX1 gene (Fig. 4C). This was confirmed by generating transgenic Pt4 lines in which the LHCX1 gene was overexpressed (OE). Several lines were isolated, and it was found that enhanced levels of LHCX1 expression could partially rescue the NPQ capacity, in the absence of any alteration in XC pigments with respect to the wild-type Pt4 (Fig. 4B).

**Table 1. Photosynthetic parameters in wild types and transgenic lines with a modulated LHCX1 content**

<table>
<thead>
<tr>
<th>Strain</th>
<th>PSII efficiency (Fv/Fm)</th>
<th>Photosynthetic electron flow (ΦPSII)</th>
<th>PSII absorption cross section (photons PSII−1·s−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt1</td>
<td>0.57 ± 0.01</td>
<td>0.32 ± 0.02</td>
<td>185 ± 7</td>
</tr>
<tr>
<td>lhcx1a</td>
<td>0.59 ± 0.02</td>
<td>0.32 ± 0.03</td>
<td>194 ± 6</td>
</tr>
<tr>
<td>lhcx1b</td>
<td>0.61 ± 0.02</td>
<td>0.31 ± 0.01</td>
<td>186 ± 2</td>
</tr>
<tr>
<td>lhcx1c</td>
<td>0.56 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>173 ± 9</td>
</tr>
<tr>
<td>Pt4</td>
<td>0.63 ± 0.01</td>
<td>0.35 ± 0.02</td>
<td>155 ± 6</td>
</tr>
<tr>
<td>Pt4OE1</td>
<td>0.59 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>184 ± 8</td>
</tr>
<tr>
<td>Pt4OE2</td>
<td>0.61 ± 0.01</td>
<td>0.32 ± 0.01</td>
<td>166 ± 12</td>
</tr>
</tbody>
</table>

PSII efficiency (Fv/Fm = (Fm–Fo)/Fm) and photosynthetic electron flow (Fm−Fs)/Fm; (36) were calculated from minimum (Fo), steady state (Fs) and maximum fluorescence emission (Fm and Fm′, measured in dark and light exposed cells, respectively). PSII absorption cross-section is the number of photons absorbed by a single PSII per unit of time. This parameter is evaluated from the rate of fluorescence induction upon inhibition of PSII with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) as 1/t, where t is the time at which variable fluorescence (F−Fo) has reached 2/3 of its maximum value. SE is relative to three independent measurements.
and C). These studies provide a definitive confirmation for the link between the abundance of the LHCX1 gene product and the capacity for NPQ in diatoms.

**Discussion**

Unlike *Chlamydomonas* and *Ostreococcus* (15), *P. tricornutum* possesses a LHCSR gene family member, LHCX1, which is highly expressed in low light-acclimated cells and is not further induced by high light stress. Its product rapidly accumulates upon a dark-to-light shift and then slowly decreases during a 12:12 h light:dark cycle, correlating with a decrease in NPQ capacity. Changes in LHCX1 levels are directly related to the ability of cells to quench excess energy (for a given amount of DT), suggesting that the function of this gene closely resembles that of the plant PSII subunit Pt. In higher plants, PsbS has a dual role: it triggers the onset of NPQ by sensing luminal pH changes (via protonation of two conserved glutamic acid residues), and it amplifies fluorescence quenching in a concentration-dependent manner (9). In diatoms, the LHCX proteins do not share these acidic amino acids (Fig. S1 B and C), questioning their role as pH sensors. In agreement with a different modulation of NPQ by the proton gradient in diatoms, previous data have shown that pH changes modulate fluorescence quenching in *P. tricornutum* only through their control on the turnover of the XC enzymes (22). On the other hand, it is clear from our data and previous evidence that both PsbS and LHCX/LHCSR proteins share the capacity to amplify quenching. It is tempting, therefore, to propose that this function could have been the ancient property of these proteins. Consistent with this, NPQ in *Chlamydomonas* shows the same pH modulation as in plants, but relies on LHCSR proteins (15) that are extremely rich in conserved acidic amino acids when compared with the *P. tricornutum* counterparts (Fig. S1 B and C). Sequence analysis reveals that these amino acids are confined to an additional protein domain in *Chlamydomonas*, which is absent in the diatom orthologs. Assuming a similar topology for LHCSR and LHCII (23) proteins, the “extra” domain of *Chlamydomonas* LHCSR should be localized in the thylakoid lumen, where it could confer the observed pH sensitivity to NPQ.

Previous studies have revealed the existence of different NPQ effectors (PsbS and LHCSR) and different xanthophyll cycles (violaxanthin/zeaxanthin or diatoxanthin/diadinoxanthin), having different efficiencies and ΔpH requirements (8, 9). This study, as well as recent findings in *Chlamydomonas* and moss (15, 24), allows pinpointing the key role of the molecular NPQ effectors, showing that efficient photoprotection in different environments is achieved using diverse NPQ machineries: PsbS (9), LHCSR–LHCX (refs. 15 and 15 this work), or both (24). Several studies have shown changes in the expression pattern of LHCX genes depending on light conditions (refs. 16–18 and this work). However, our work underlines that the presence of the constitutive LHCX1 gene product is essential for proper light acclimation in *P. tricornutum*, its decrease resulting in reduced growth capacity in low light, high light, and intermittent light (Fig. S4). We believe that besides preventing photooxidation at high light, LHCSX1 could also influence the ability of *P. tricornutum* to acclimate to nonstressful light regimes by modulating light acclimation during repeated diurnal dark-to-light shifts during exponential growth. This would mainly stem from changes in the transient NPQ response, which is observed at the onset of illumination at moderate light intensities (Fig. S6).

In principle, the recent hypothesis that in *Thalassiosira pseudonana* LHCX1 may play a structural role in the PSII–FCP complex (18) could also be consistent with the reduced growth observed in the *P. tricornutum* lhcx1 knock-down lines in nonstressful light conditions. However, this hypothesis is difficult to reconcile with our findings that both the antenna size and the photosynthetic activity are unmodified in the lhcx1 cells when compared with their wild-type counterpart (Table 1 and Fig. S34).

![Fig. 4. Pt4 is a natural NPQ variant displaying altered LHCX expression. (A) Analysis of 10 P. tricornutum ecotypes isolated from different locations (Left), Maximum NPQ as a function of growth temperature (Right). Cells were grown at the indicated temperatures for at least 3 wk. Samples were collected 2 h after the onset of illumination and NPQ was measured as in Fig. 1. (B) NPQ (Left and Center) in Pt1, Pt4, and two Pt4 transgenic lines overexpressing LHCX1. Right shows their DES values. (C) LHCX1 mRNA levels by qRT-PCR using RPS as reference gene (Left) and protein accumulation (Right). A total of 50 μg of total protein extracts was used in the Western blot analysis for Pt4 and Pt1. To better highlight differences, 20 μg has been loaded for the Pt4 and Pt4 overexpressing lines (OE1 and OE2). Proteins were detected with antibodies against the LHCSR/LHCX, and the photosystem II subunit D2 was used as a loading control.](image)
The identification of a natural NPQ variant that displays reduced LHCX1 expression (Pt4) indicates that NPQ effectors have also been targeted for adaptive evolution to specific environments. Genetic analyses indicate that Pt4 is the most diverse P. tricornutum strain (20), and the lower NPQ capacity may reflect its adaptation to high latitudes, with exposure to lower light intensities and to less drastic diurnal light variations. It is possible, therefore, that this ecotype may represent the result of a favorable mutation event in the Baltic environment. Because of the very recent flooding of this basin after the last glaciation and its strong isolation due to topographic and thermohaline constraints (25), this mutation could have been confined within this area. Interestingly, although similar variation in NPQ has also been observed in Arabidopsis ecotypes (26), no correlation with Pb5 levels has been found.

In conclusion, our data challenge the long-standing dogma that the peculiar characteristics of NPQ in diatoms are due solely to the presence of a novel xanthophyll cycle. Our molecular study in P. tricornutum unveils the key role of LHCX1 as a molecular gauge controlling quantitatively the level of NPQ. The constitutive presence of this protein in cells acclimated to nonstressful light conditions could provide diatoms with a machinery capable of anticipating sudden changes in the underwater light field. This property could have offered a selective growth advantage in turbulent water (9). Diatoms LHCX genes appear to be widely dispersed in the ocean (http://camera.calit2.net/), further reinforcing our tenet that they have contributed to the ecological success of diatoms in ocean environments.

Materials and Methods

Cell Cultures. Axenic P. tricornutum cells were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton and grown in f/2 medium (27) at 19 °C in a 12-h photoperiod. Cells were grown under low light (30 or 70 μmol photons m−2 s−1) and collected during the exponential growth phase. The experiments shown in Fig. 1, cells were first acclimated to 70 μmol photons m−2 s−1 for 2 h and then shifted to 600 μmol photons m−2 s−1 white light for 1 h. Cells used for data presented in Fig. 3A and Fig. S6 were grown at the indicated temperatures, light intensities, and salinities for at least 3 wk before measurements. These parameters were varied within a range consistent with possible variation at the ocean surface (21, 28).

Construction of Vectors for Gene Silencing and Overexpression. Vectors for antisense constructs were generated using standard molecular cloning procedures (29). Vectors containing antisense fragments from the different LHCX genes were generated in a vector bearing a phleomycin resistance cassette, as described in SI Materials and Methods. Vectors were introduced into wild-type P. tricornutum strains (Pt1 and Pt4 ecotypes) by microparticle bombardment using a BiolisticPDS-1000/He Particle Delivery System (Bio-Rad) (30). BLAST searches in the P. tricornutum database (31) and from Alix Boulouis for sampling during the time-course experiments is also acknowledged. The project was supported by grants from the JST-Centre National de la Recherche Scientifique (CNRS) collaborative project on Marine Genomics and Marine Biology, the Agence Nationale de la Recherche (ANR) Phytadapt project (to G.F. and C.B.), the Human Frontier Science Program (HFS) Career Development Award (00142006), the FWF Marie Curie Initial Training Network (ITN) (COS1; 215174) and the Action Thématique et Initiative sur Programme (ATIP) award (2009) from CNRS (to A.F.). P.C. is an Fond de la Recherche Scientifique–Fond National de la Recherche (FRS-FNRS) research associate.


